ANALYSIS OF LSAMP GENE AS A TUMOR SUPPRESSOR IN NEUROBLASTOMA

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BY
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DECEMBER 2009

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

ANALYSIS OF LSAMP GENE AS A TUMOR SUPPRESSOR IN NEUROBLASTOMA

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M.Sc. in Molecular Biology and Genetics

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Neuroblastoma constitutes approximately 10 % of all childhood tumors with a worldwide considerable morbidity and mortality. Frequently, in children under the age of 1, it can spontaneously regress and transform into a benign tumor. However, in children older than age of 1 the disease often behaves aggressively and metastasizes to other organs. This unpredictable behavior of unknown origin makes therapeutic applications ineffective.

Limbic system associated membrane protein gene (*LSAMP)* functions in neurite growth, axonal guidance and acts as a cell adhesion and recognition molecule. Recent studies revealed its association to several cancer types and proposed a potential tumor suppressor role. Markers in the *LSAMP* gene region were also shown to be homozygously deleted in neuroblastoma. In the framework of this study, we investigated *LSAMP* gene in respect of its potential tumor suppressor role in neuroblastoma. 6 clinical patient samples and 2 neuroblastoma cell lines were studied via PCR methodology to detect any loss in *LSAMP* gene. Immunohistochemistry (IHC) was applied to 6 neuroblastoma tissue sections to determine protein level changes of *LSAMP*. Moreover, expression analysis in a set of brain tumors was performed. As a result of these efforts, one possible LOH and one homozygous deletion in two different patients were observed. Low levels of LSAMP protein in all of the tumor samples compared to controls were recorded.

Downregulation of *LSAMP* in brain tumors was detected. Based on these results, *LSAMP* is suggested as a candidate tumor suppressor in neuroblastoma and in broader aspect for nervous system tumors.

ÖZET

LSAMP GENİNİN NÖROBLASTOMALARDA TÜMÖR BASKILAYICI OLARAK ANALİZİ

ATIL ÇAĞDAŞ SAYDERE

Moleküler Biyoloji ve Genetik Bölümü Yüksek Lisansı

Danışman: Assist. Prof. M. Cengiz Yakıcıer

Aralık 2009, 59 sayfa

Nöroblastoma dünya çapında önemli morbidite ve mortaliteye sahip olup tüm çocukluk çağı tümörlerinin yaklaşık %10'unu oluşturmaktadır. Sıklıkla, 1 yaş altındaki çocuklarda, kendiliğinden gerileyerek ve iyi huylu bir tümör haline dönüşmektedir. Ancak, 1 yaş üzeri çocuklarda genellikle agresif davranır ve diğer organlara metastaz olur. Kaynağı bilinmeyen bu beklenmedik davranış tedavi uygulamalarını etkisiz hale getirir.

Kromozom bölgesi 3q13.3 üzerinde bulunan limbik sistem ilişkili membran proteini geni *(LSAMP)* sinir hücrelerinin büyümesinde ve akson rehberliğinde görevlidir. Ayrıca, bir adezyon ve hücreler arası tanıma molekülü gibi davranır. Son yıllarda yapılan çalışmalarda çeşitli kanser türleriyle ilişkisi gösterilmiş ve potansiyel bir tümör baskılayıcı rolü önerilmiştir.Ayrıca *LSAMP* geni bölgesindeki markörlerin homozigot kayıplara uğradığı gösterilmiştir. Bu çalışma çerçevesinde, *LSAMP* geninin nöroblastomada olası bir tümör baskılayıcı rolü araştırıldı. 6 klinik hasta numunesi ve 2 nöroblastoma hücre hattı PCR yöntemi ile LSAMP gen kaybını ortaya çıkarmak için çalışıldı. 6 nöroblastoma doku kesitinde LSAMP protein düzeyindeki değişiklikleri belirlemek için immunohistokimya (İHK) uygulandı. Bir grup beyin tümöründe ifade analizi yapıldı. İki farklı hastada bu çabalar, olası bir LOH ve bir homozigot kayıp sonuçları olarak gözlemlendi. Kontrol grubuna göre tüm tümör örneklerinde LSAMP proteinin düşük seviyeleri kaydedildi. Beyin tümörlerinde

LSAMP ifadesinde azalma belirlendi.Bu sonuçlara dayanarak, *LSAMP* tümör baskılayıcı adayı olarak nöroblastomada ve daha geniş anlamda sinir sistemi tümörlerinde önerilmektedir.

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ABBREVIATIONS

ACTB:	B-actin
bp:	Base Pair
cDNA :	Complementary DNA
CGH:	Comparative Genomic Hybridization
ΔH2O:	Double Distilled Water
DM:	Double Minutes
DNA:	Deoxyribonucleicacid
dNTP:	Deoxyribonucleotide Triphosphate
EtBr:	Ethidium Bromide
EDTA:	Ethylenediaminetetraacetic acid
FBS:	Fetal Bovine Serum
FISH:	Fluorescent in situ Hybridization
g:	Gram
GAPDH:	Glyceraldehyde-3-Phosphate Dehydrogenase
HCC:	Hepatocellular carcinoma
HSR:	Homogenously Staining Regions
IHC:	Immunohistochemistry
LOH:	Loss of Heterozygosity
LSAMP:	Limbic System Associated Membrane Protein
min:	Minute
ml:	Mililiter
mM:	Milimolar
MP-PCR:	Multiplex PCR
mRNA:	Messenger RNA
MYCN:	v-myc Avian Myelocytomatosis Viral Related Oncogene, Neuroblastoma Derived
NaCl:	Sodium Chloride
PBS:	Phosphate Buffered Saline
PCR:	Polymerase Chain Reaction

pm:	Picomole
Q-RT-PCR:	Quantitative Real Time PCR
RNA:	Ribo Nucleic Acid
Rpm:	Revolutions Per Minute
Sec :	Second
TAE:	Tris Acetate EDTA Buffer
Tris:	Tris (Hydroxymethyl)-Methylamine
UV:	Ultraviolet
w/v:	Weight/Volume
µg:	Microgram
µl:	Microliter
µm:	Micrometer

1 INTRODUCTION

Neuroblastoma was first identified and characterized as a tumor by Virchow in 1864 and by Marchand in 1891, respectively (Hayes PA, 1989; Voute PA, 1984). Since their identification, the tumor has succeeded to stimulate interests of the researchers by its distinct characteristics. The neuroblastoma tumor tissues either regress spontaneously as observed especially in infants or they mature into benign tumors called ganglioneuroma (Brodeur and Castleberry, 1993). However, it is almost impossible to predict the behavior and most children over 1 year of age already have tumor metastasized into other organs at the time of diagnosis (Brodeur, 2003). The heterogeneous behavior of the disease prevented comprehensive clinical studies and resulted in poor prognosis until studying the tumor with the tools of molecular genetics and biochemistry.

1.1 Epidemiology

Neuroblastoma is the most common extracranial solid tumor seen in pediatric population. It accounts for 8-10 % among all childhood cancers and it is the most common diagnosed cancer type for infants (Gurney et al., 1997). Neuroblastoma is diagnosed in United States and Canada one case in 7000 live births per year and approximately 700 new cases per year are observed (Brodeur and Castleberry, 1993). In Europe compared to USA and Canada more than two times higher, 1500 cases occur per year (Gao et al., 1997; Spix et al., 2006). Among all cancers diagnosed in European and USA infants, neuroblastoma accounts for about 28% (Gurney et al., 1997; Spix et al., 2006). Almost the same incidence rate for the rest of the world according to reported cases are observed (Brodeur and Castleberry, 1993). The incidence has a peak before the age 1 and the median age of the patients diagnosed with neuroblastoma is 18 months (Brodeur and Castleberry, 1993; Heck et al., 2009).

There is an equivalent or slightly higher prevalence of the disease in boys than girls in most countries. There is no difference between the ethnic groups for the incidence of the disease (Gao et al., 1997; Ries et al., 2005; Spix et al., 2006).

1.2 Pathogenesis and Aetiology

1.2.1 Pathogenesis

Neuroblastoma is a malignant tumor originating from the neural crest cells which arise in the third to fourth week of embryonic development. Some pluripotent cells or neuroblasts of this layer can form the components of the sympathetic nervous system by differentiating into sub populations (Fig.1). These cells invaginate and migrate along the neuraxis and accumulate in sympathetic ganglia, adrenal medulla and different sites of the bone and soft tissue (Gray H, 2005). The distribution of these cells into the sub regions of the sympathetic system correlates with the primary diagnosis sites of the neuroblastoma. In most cases, approximately 40%, the origin side of the tumor is one of the adrenal glands or in other cases it is observed in the chest, abdomen and pelvic (Gao et al., 1997; Ries et al., 2005).

Among other known human malignancies, neuroblastoma distinguishes itself by an enigmatic behavior in which some cells undergo a spontaneous regression from a malign state to a benign state while others persistently progress (Brodeur and Maris, 2006) . The tumor may undergo differentiation and/or apoptosis in patients under 1 year of age in contrast with patients older than 1 year of age where it shows an aggressive behavior and leads to death (Brodeur, 2003; Nakagawara, 1998).

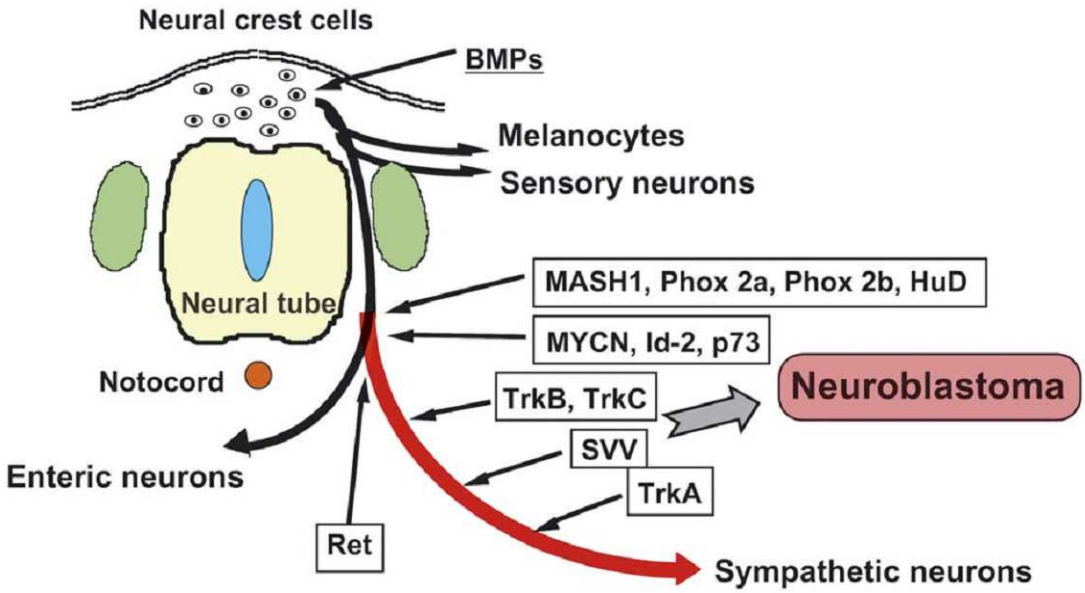


Figure 1: The lineages derived from neural crest and the origin of neuroblastoma. (Nakagawara and Ohira, 2004)

In order to facilitate diagnosis and prognosis of the disease a classification system called Shimada System which classify patients into three risk groups; low, intermediate and high was created in 1984 (Shimada et al., 1984). It was used to determine the curability of the patient ; low risk and intermediate risk patients which usually comprise infants have a higher chance of cure compared to high risk group patients who have poor diagnosis and outcome (Maris, 2005). The majority of the patients older than 18 months have the disease metastasized to lymph nodes, liver, bone and bone marrow but despite the intensive therapies followed by autologous bone marrow transplant, more than half of the patients cannot survive (Spix et al., 2006)

The recent advances allowed the disease to be re-classified into different stages and risk groups by using factors like differentiation state, *MYCN* amplification, age, Schwannian stroma content and mitosis-karyorrhexis index at the time of diagnosis (Maris et al., 2007; Shimada et al., 1999). The efforts of International Neuroblastoma Risk Group (INRG) developed a classification system in modifying the previously used Schimada System. According to this classification schema the tumors are

categorized into three risk groups; low, intermediate and high and different stages of the disease is defined from 1 to 4 where 4 represents the metastasized tumor (Maris et al., 2007). One stage called 4s which is special for the patients younger than 1 year of age and seen nearly 5% of the patients is also described. In this case, the tumor is spread to liver, skin or bone marrow but show a spontaneous regression (D'Angio et al., 1971).

1.2.2 Aetiology

The sporadic occurrence of the disease makes it challenging to study and there is not much known about the aetiology of the disease but as a common reason in tumorigenesis environmental effects are thought to play a role for the development of the disease (Brodeur, 2003). Among these environmental factors, using medications like pain killers, exposure to electro-magnetic fields, the use of sex hormones and vitamins has been hypothesized for the aetiology of the disease (Carachi, 2002). In recent studies maternal alcohol consumption, paternal exposure to nonvolatile and volatile hydrocarbons, wood dusts and solders, use of diuretics and low birth weight are suggested to be positively associated with the disease (Heck et al., 2009).

1.3 Genetic Aberrations in Neuroblastoma

The molecular pathology of neuroblastoma is still poorly understood. However, common genetic alterations have been observed in patients diagnosed with neuroblastoma and these alterations are used for risk stratification. The best characterized genetic alterations include amplification of the proto-oncogene *MYCN*, gain of chromosome arm 17q and losses of 1p, 3p, and 11q. Based on these aberrations, neuroblastoma is classified into three genetic sub groups. Beside *MYCN* on chromosome arm 2p which was previously identified as an oncogene, further efforts are focused on identifying any candidate tumor suppressor genes or

oncogenes located within the regions of loss or gain, respectively. Until now, no new genes that drive neuroblastoma development and progression have been successfully characterized.

1.3.1 Genomic Gains:

1.3.1.1 Ploidy:

DNA content is an important prognostic marker for the neuroblastoma patients. Although in most cases tumors have diploid karyotypes, a distinct case for lower stage neuroblastoma patients exist as they may often have hyperdiploid or near triploid (Kaneko et al., 1987). Thus, during prognosis the karyotype analysis of neuroblastomas is performed to determine the ploidy status and having near-triploidy is more favorable for the patients associated with longer survival. However, it is more significant for infants compared to children older than 1 year of age. The reason behind is that less aggressive tumlors have a major defect in mitosis which results in whole chromosomal gains and losses where aggressive tumors additionally imperfect in genomic stability which ends in structural rearrangements (Brodeur, 2003; Look et al., 1984).

1.3.1.2 *MYCN* Genomic Amplification:

The well known associated marker of neuroblastoma is the genomic amplification of *MYCN* which occurs in 20% of patients and strongly correlates with higher stages of the disease and poor outcome (Brodeur and Seeger, 1986; Schwab et al., 2003; Shimada et al., 1999). *MYCN* gene is located on chromosome 2p24 locus and it was identified in neuroblastoma cell lines as an amplified DNA sequence homologous to proto-oncogene *c-MYC*. The amplified region that contains *MYCN* chromosome

2p24 locus form double minute chromosomes (DM) and they are integrated linearly to random chromosomes resulting in homogenously staining regions (HSR) that may contain copies up to 500 (Schwab et al., 1983) (Fig. 2). Rather than a mutation in this amplified region, it is suggested that overexpression of wild type *MYCN* leads to tumorigenesis as overexpressing *MYCN* in neural crest of transgenic mice can develop neuroblastomas and decreasing *MYCN* mRNA levels by using antisense *MYCN* can induce differentiation of human neuroblastoma cell lines (Schmidt et al., 1994; Weiss et al., 1997).

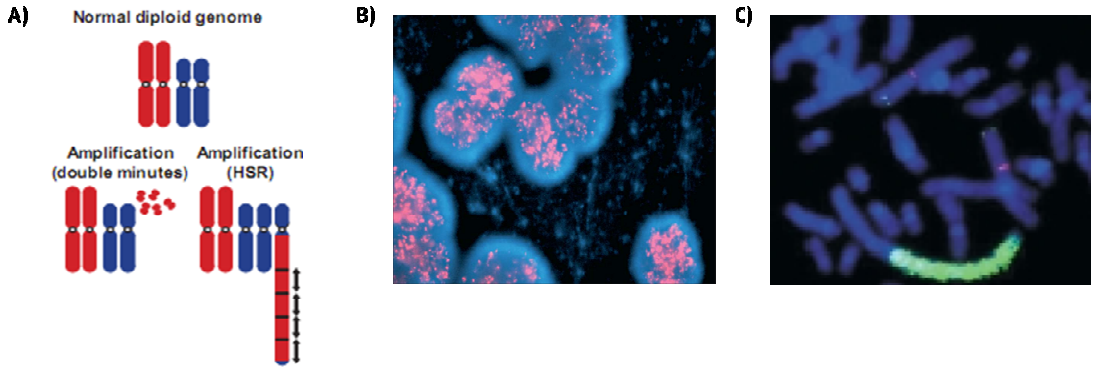


Figure 2: MYCN amplification pattern; A) Amplification patterns of double minutes (DM) and homogenously staining regions (HSR) are observed in solid tumors (Albertson et al., 2003) B) FISH image of MYCN amplification presented in DM (Maris et al., 2007) C) HSR for MYCN amplification in neuroblastoma cell line NGP (Schwab et al., 2003)

1.3.1.3 Gain of 17q:

Extra copies of chromosome arm 17q are observed in more than half of the neuroblastomas. The gain of 17q may occur independently but more often results from unbalanced translocation events with chromosome 1p or 11q and associated with poor outcome (Bown et al., 1999; Caron, 1995). The gene dosage effect of one or more genes in extra copies is thought to provide a selective advantage to cells resulting in aggressive phenotype (Lastowska et al., 2002). Although there is no exact site of breakpoint, the region of 17q22-qter has been suggested as carrying

genes that promotes cell survival (Van Roy et al., 1997). The most of the genes have not been identified yet but among the known genes overexpression of BIRC5 (survivin) which encodes an inhibitor of apoptosis proteins has been proposed for the reason of aggressive phenotype seen in gain of 17q (Islam et al., 2000). In some cases segmental gains of 1q, 5q and 18q are also observed but their characterizations still remain in question (Schwab et al., 2003).

1.3.2 Genetic Deletions and Allelic Losses:

1.3.2.1 Loss of 1p:

The most common loss of heterozygosity (LOH) seen in neuroblastoma patients is the loss of 1p which is seen about 30-35% of the cases (Brodeur, 2003). Deletions in chromosome region 1p correlate with the advanced stages of the disease and frequently associated with *MYCN* amplification and unbalanced translocation with 17q, t (1; 17) (Caron, 1995). Studies identified the smallest region that overlaps in tumors with loss of 1p as the locus 1p36 and loss of putative tumor suppressors in this region is thought to be responsible for disease progression (Caron et al., 2001). However, there is no consensus about the role of this locus independent of other factors as there are inconsistent data presented by different groups.

1.3.2.2 Loss of 11q:

Approximately 35% of the diagnosed neuroblastoma patients have deletions at chromosome region 11q. Translocations involving 11q21 and 11q22, deletion at 11q23, inversion at 11q21-11q23 and allelic losses are observed (Brodeur and Maris, 2006). In most of the cases allelic losses are reported and it is negatively correlated with *MYCN* amplification. Deletion in 11q is frequently found in neuroblastomas

without *MYCN* amplification (Guo et al., 1999; Plantaz et al., 2001). However, the loss at this region associates with the advanced stages of the disease and poor prognosis. The existence of tumor suppressor genes in this region is thought to be responsible for malignant progression upon inactivation by allelic losses in *MYCN* single copy neuroblastomas (van Noesel and Versteeg, 2004).

1.3.2.3 Losses at Other Chromosomes:

Beside chromosome regions 1p and 11q, allelic losses at chromosome arms 2q, 3p, 4p, 9p.12p, 14q, 15q, 16p and 19q are observed in lower frequencies. Several genes are identified in these regions as tumor suppressors or important regulators of cellular functions, but further investigation are needed for their prognostic significance (Table 1).

Chromosomal Locus	Gene Name	Gene Function
1p36.2-p36.3	?	Tumor suppressor
1p13	*NGF*	Neurotrophin ligand for *NTRKI*
1q23-q31	*NTRK1 (TRK-A)*	Receptor tyrosine kinase
2p12-13	*MAD*	May regulate *MYCN*
2p241	*MYCN*	Proto-oncogene
2p24	*DDXI*	RNA helicase/oncogene
3p	?	Tumor suppressor
4p	?	Tumor suppressor
7q21	*PGY1 (MDR1)*	Multi drug resistance
9q22.1	*NTRK2 (TRK-B)*	Receptor tyrosine kinase
11 p13	*CD44*	Integrin/metastasis suppression
11 p13	*BDNF*	Neurotrophin ligand for *NTRKI*
11 q23	?	Tumor suppressor
12p1 3	*NTF3 (NT-3)*	Neurotrophin ligand for *NTRK3*
14q23	*MAX*	Regulates *MYCN*
14q23-qter	?	Tumor suppressor
15q24-q25	*NTRK3 (TRK-C)*	Receptor tyrosine kinase
16p13.1	*MRP*	Multi drug resistance
17q22	*NMEI*	Nucleoside kinase/metastasis suppression
17q23-qter	?	Oncogene
18q21.1	*DCC*	Tumor suppressor
18q21 3	*BCL2*	Apoptosis suppression
19	*NTF4 (NT-4)*	Neurotrophin ligand for *NTRK2*

Table 1: Proven or speculated chromosomal loci involved in neuroblastoma tumorigenesis (Maris and Matthay, 1999)

1.3.3 Abnormal Expression of Neurotrophin Receptors:

Neurotrophins are essential soluble factors needed during neural development and majorly include nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). Neuroblastomas as originating from neural crest have alterations in normal neural development pathway resulting in malignant transformation (Fig.3). However, in cases of spontaneous regression where the cells start to differentiate and apoptosis takes place, the recovery of neuronal development pathway occurs (Maris and Matthay, 1999). The signaling cascades are regulated through the Trk family of tyrosine kinases and *TRKA*, *TRKB* and *TRKC* have been defined as key receptors in this pathway. Specific receptor–ligand interactions occur; *TRKA* is the receptor for ligand NGF, *TRKB* serves for both BDNF and NT-4, and *TRKC* is the receptor for NT-3 (Nakagawara and Brodeur, 1997). The expression of *TRKA* is highly associated with favorable tumors and spontaneous regression where expression of *TRKB* is usually observed in aggressive tumors. The *TRKB*/BDNF signaling promotes cell survival enhancing angiogenesis and drug resistance. *TRKC* is also found expressed in lower stage tumors and is not expressed in *MYCN* amplified tumors (Matsumoto et al., 1995; Nakagawara, 1998).

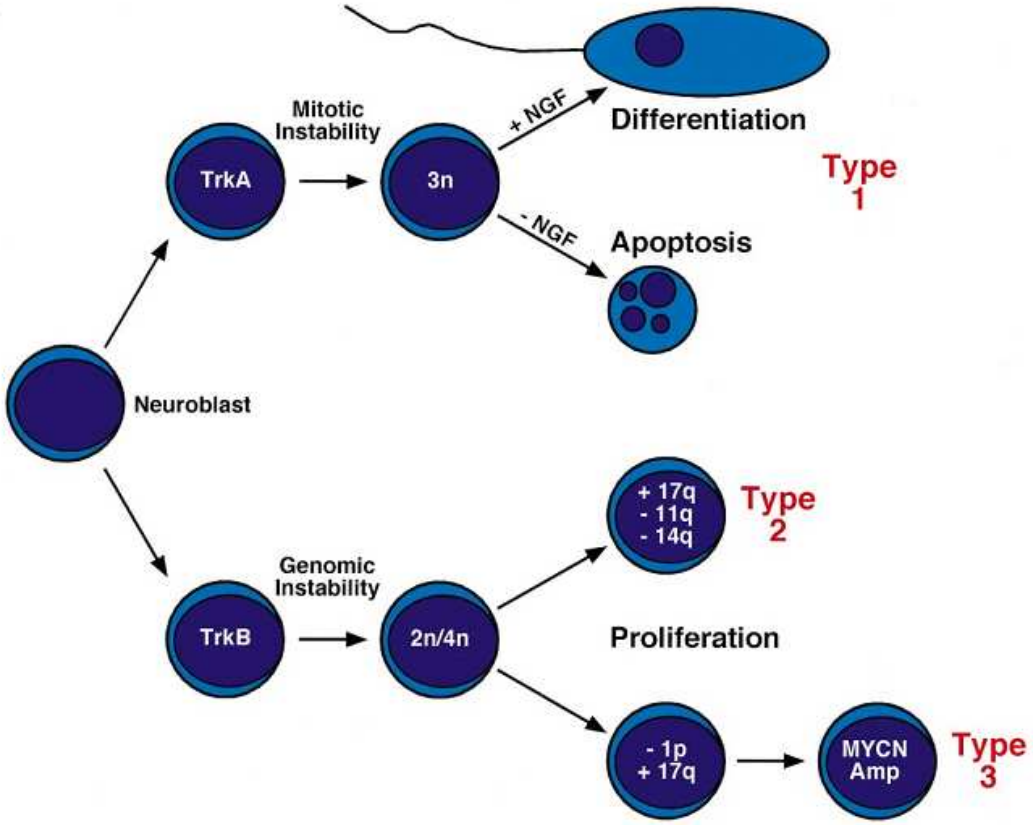


Figure 3: Schematic presentation of genetic alterations in neuroblastoma and classification into different types (Maris and Matthay, 1999)

1.4 LSAMP gene:

*LSAMP (*Limbic System Associated Membrane Protein*)* encodes a neuronal surface glycoprotein protein and belongs to a family of genes called IgLON consisting of *LSAMP (LAMP), OPCML*/*OBCAM* (Opioid Binding Protein/Cell Adhesion Molecule Like), NTM (Neurotrimin) and *NEGR1*/*KILON* (Neuronal Growth Regulator 1) (Funatsu et al., 1999; Pimenta et al., 1995). The IgLONs are identified as immunoglobulin subfamily of glycosylphosphatidylinositol-anchored cell adhesion molecules. The expressions of these genes are distributed in cortical and subcortical regions of the limbic system (Levitt, 1984; Struyk et al., 1995). During nervous system development, specific connections between neuronal cell populations

must be established correctly for proper growth and the axonal targeting molecules play a major role during this process. *LSAMP* together with other IgLONs is highly conserved among higher organisms and it mediates neuronal growth and axon targeting as well as acting in cell-cell recognition and cell adhesion (Zhukareva and Levitt, 1995).

LSAMP is located on chromosome 3q13.31-q21 locus in the human genome (Fig.4) (Pimenta et al., 1998). It has seven exons with six alternative forms identified recently and encodes a plasma membrane protein with three Ig-like C2 type (immunoglobulin-like) domains and one glycosylphosphatidylinositol anchor. Microarray data reveal that *LSAMP* is highly expressed in cortical and subcortical regions of limbic system as well as tissues of parasympathetic and sympathetic nervous system (Fig.5). Markers in this region were also shown to be homozygously deleted in neuroblastoma cell lines (Caren et al., 2008).

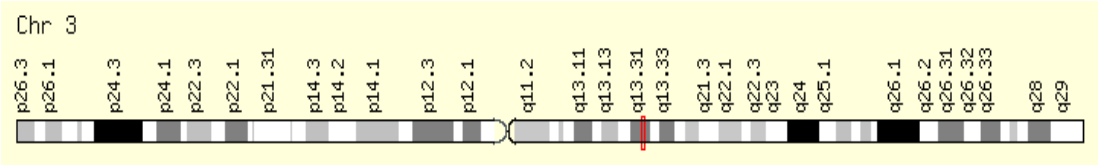


Figure 4: Location of LSAMP gene in long arm of chromosome 3.

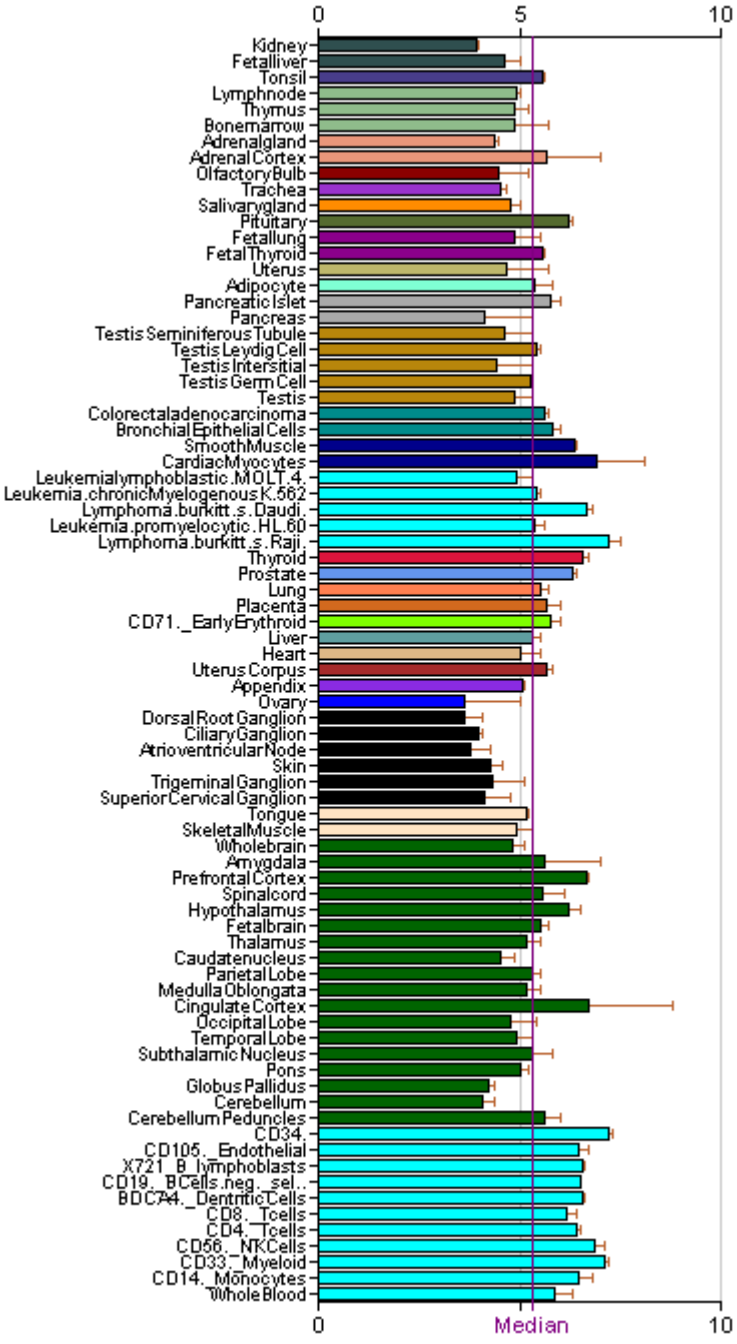


Figure 5: Expression of LSAMP in different tissues (Data from Genomics Institute of the Novartis Research Foundation)

Until recently, there was not any data associating *LSAMP* to cancer and suggesting it as a tumor suppressor gene. However, low expression and polymorphism of *LSAMP* was found to be associated with behavioral disorders like hyperactivation, anxiety and panic in studies done with mice (Catania et al., 2008; Koido et al., 2006; Maron et al., 2006). Moreover, a study by Must et al. (2008) correlated *LSAMP* polymorphism to completed male suicide (Must et al., 2008). Wang. et al. (2008) proposed that genetic variations in *LSAMP* are associated with left main coronary artery disease (Wang et al., 2008)

The first hypothesis for *LSAMP* to be a tumor suppressor gene came from the study of Chen et al. (2003) in familial clear cell renal cell carcinoma (CCRCC) (Chen et al., 2003). A translocation, t (1; 3), involving *NORE1* and *LSAMP* as breakpoint spanning genes was identified in CCRCC. Although no mutation was observed for *LSAMP*, the promoter of *LSAMP* was found as hypermethylated and silenced in CCRCC cell lines. Moreover, *LSAMP* was also found to be methylated in 26 % of colorectal cancers by additional experiments. In several tumors independent of methylation status loss of heterozygosity (LOH) of *LSAMP* was observed. Increasing expression of *LSAMP* in samples where it is methylated, had an inhibitory effect on cell proliferation which supported the hypothesis of *LSAMP* as a candidate tumor suppressor gene in CCRCCs (Chen et al., 2003).

In a broad study investigating the status of IgLON family genes in epithelial ovarian cancer, *LSAMP* was shown to be a potential tumor suppressor gene in epithelial ovarian cancer (Ntougkos et al., 2005). Besides being differentially expressed between normal and tumor, *LSAMP* expression was also found to be associated with differentiation status as it is highly expressed in well or moderate tumors (Ntougkos et al., 2005) Among the IgLON family members, *OPCML* was also suggested as a tumor suppressor gene based on data obtained from epithelial ovarian cancer. Inactivation by LOH and hypermethylation was frequently observed in tumor

samples. Moreover, decreased expression and epigenetic inactivation of *OPCML* has been shown in brain tumors, multiple carcinomas and lymphomas (Cui et al., 2008; Reed et al., 2007) .

Recently, two different groups identified *LSAMP* deletions in osteosarcomas (Kresse et al., 2009; Yen et al., 2009a). Kresse et al. (2009) showed that chromosomal region containing *LSAMP* gene is frequently deleted in osteosarcoma tumor samples and cell lines by using CGH array analysis. Moreover, at the expression level, low expression of *LSAMP* in tumors was shown to be correlated with poor survival (Kresse et al., 2009). Yen et al. (2009) also identified chromosomal aberrations in the region of 3q13.31 where *LSAMP* is located to be associated with progression of osteosarcomas. Homozygous or heterozygous deletions of *LSAMP* in primary osteosarcomas were detected and further supported with reduced expression levels of *LSAMP* in the same tumors (Yen et al., 2009b).

2 AIM:

Clinical studies are not sufficient for good prognosis in advanced stages of neuroblastoma which has a poor survival. Elucidating the molecular biology of neuroblastoma is a crucial step for improved prognosis and therapies. Inactivation of one or more tumor suppressor gene(s) in a neuroblast cell has been proposed for the initial step of the neuroblastoma genesis. Identification of tumor suppressor genes has a great potential for revealing the mechanism of tumor development and further investigation of new therapeutic approaches. Previous efforts identified several genetic alterations that may be used as prognostic markers in neuroblastoma. Among these alterations the amplification of *MYCN* which is identified in 20% of the cases has been used widely since its detection as the first strong indicator of poor outcome. However, *MYCN* amplification or other aberrations are not enough in classifying patients into low or high risk groups because of the heterogeneity of neuroblastoma.

Since neuroblastoma is neural crest originated, it is rational to inspect genes that drive neural development. IgLON family members (*LSAMP, OPCML*, *NTM*, and *NEGR1*) are well characterized for their function in developing neural system. Furthermore, they function as cell adhesion molecules whose roles in tumorigenesis are well established. Recently, several studies suggested two members of this family, *LSAMP* and *OPCML*, as potential tumor suppressors for epithelial ovarian cancer. Loss of *OPCML* is further associated with brain tumors. *LSAMP* is also linked to clear cell renal cell carcinoma and osteosarcomas. Moreover, homozygous deletions of *LSAMP* marker in neuroblastoma cell lines were shown.

In the light of these data and necessitate for improving prognosis in neuroblastoma, this study aims to identify a novel candidate tumor suppressor; *LSAMP*, which we hypothesize to be a gene that plays a role in neuroblastoma tumorigenesis.

3 MATERIALS AND METHODS:

3.1 Materials:

3.1.1 Chemical Solutions and Reagents:

Ethidium Bromide (EtBr):

10mg/ml in water (stock solution)

30ng/ml (working solution)

50X TAE Buffer:

121 g Tris Base

28.55 ml Acetic Acid (Glacial)

18.6 g $Na_2EDTA.H_2O$ (Triplex) (Added in last step to ease dissolving)

ΔH_2O is added to 500 ml.

6X Loading Buffer:

30% Glycerol

0.04% Bromphenolblue

0.04% Xylene Cyanol

ΔdH_2O

2% (w/v) Agarose Gel:

50 ml 1X TAE

1 g Agarose (Prona, Basica Le Agarose)

10 mM TE Buffer:

ΔdH_2O to 400 ml

0.61 g Tris

0.15 g EDTA (m.w. 292.1)

ΔdH_2O to bring volume to 500 ml

Adjust pH to 7.5 - 8.0

10mM TE/1% Tween 20:

1 ml Tween 20 is added into 100 ml of 10mM TE Buffer

3.1.2 PCR Primers and Conditions:

3.1.2.1 Semi-Quantitative and Multiplex PCR Reactions:

3.1.2.1.1 Master Mix:

Phire™ Hot Start DNA polymerase (F-120S) and DyNAzyme™ II DNA Polymerase (F-503S) were purchased from Finnzymes's (Espoo, Finland) and were used in semi Q-PCR and Multiplex PCR reactions, respectively. Reaction buffers and MgCl2 buffers provided with the DNA polymerases were used. dNTP mix (10 mM of each nucleotide) (#R0192) of Fermentas (Burlington, Canada) and HyClone HyPure™ Molecular Biology Grade Water produced by Thermo Scientific (Waltham, USA) were used in all reactions. Primers used in the study were synthesized by IONTEK (Istanbul, Turkey).

3.1.2.1.2 Primers:

Primers for *LSAMP* and *GAPDH* were designed by using Primer3 program available online at http://frodo.wi.mit.edu/. Intron – exon boundaries were used in design of LSAMPgnf54 and LSAMPex3r primer pairs which was used for amplifying only genomic DNA of 98 bp in neuroblastoma PCR reactions. LSAMPutrFrw and

LSAMPex7cdRev primer pairs were used in brain tumors with a product size of 1167bp. A second primer pair LSAMPcdNstFrw and LSAMPcdNstRev were used further in Nested PCR reactions of brain tumors producing a band of 167 bp. *GAPDH* primers were designed for genomic amplifications and cover 143 bp. Designed primers were ordered from IONTEK (Istanbul, Turkey) and purchased in lyophilized form. All primers were hydrated to a concentration of 100 pm. The sequences of the primers are given in Table 2.

Primer Name	**Sequence (5′ to 3′)**	**Product Size**
LSAMPgnf54	CCTGGAGACACCTTACACCAAC	98bp
LSAMPex3R	ATTCAGCAGAATTCCAGGAGC	
LSAMPutrFrw	CTGAGCGAGGGAAAGAGAGA	1167bp
LSAMPex7cdRev	AGAAGGCAGAGCAGAGATGC	
LSAMPcdNstFrw	CTCCAATATCTCCTCGGATGTC	167bp
LSAMPcdNstRev	GTGATGCCAAGGATCTCCAG	
GAPDH_GENOMIC_20070206_F	ACACCCACTCCTCCACCTTT	143bp
GAPDH_GENOMIC_20070206_R	CTGAGCCAGCCACCAGAG	

Table 2: Primers Used in PCR reactions

3.1.2.2 Quantitative Real Rime PCR (Q-RT-PCR) Reactions:

3.1.2.2.1 Master Mix:

For all Q-RT-PCR reactions master mix of Bio-Rad IQ SYBR Green Supermix (California, USA) was used. Reaction steps were performed according to the manufacturer's instructions. Reaction conditions are listed in Table 7.1 & 7.2 in methods section under "Quantitative-RT-PCR Reaction" title.

3.1.2.2.2 Primers:

SybGREEN qPCR *LSAMP* primer mix was commercially purchased from Ori-Gene Company (Cat. no: HP206046). Primer sequences are not specified in respect to company's confidentiality procedure. Primers are known to amplify a region around 95 to 140 bp. Beta-actin gene (*ACTB*) was selected to use as internal control.

3.1.2.2.3 Instrument:

Stratagene Mx5000P (California, USA) instrument was used to perform Q-RT-PCR experiments.

3.1.3 Tumor Samples:

Neuroblastoma tumor samples and tissue sections were provided by Dr. Aylin Okçu Heper from School of Medicine, Department of Pathology, Ankara University. 6 neuroblastoma patients provided research materials. 2 paraffin embedded tissue sections from each of 6 patients, 12 samples in total, and 14 poly-l lysine coated tissue slides were kindly provided. 12 samples and 8 tissue slides involving 6 tumors and 2 controls were used for protein level analysis of *LSAMP* (Table 3).

Tumor Samples		Tissue Sections
12116-1		12116-1
12116-2		12116-2
28231-1		28231-1
28231-2		28231-2
3978-kon603006-1		3978-kon603006-1
3978-kon603006-2		3978-kon603006-2
3755-1		3755-1
3755-2		3755-2
13598-B3-1		13598-B3-1
13598-B3-1		13598-B3-1
1506-1		1506-1
1506-2		1506-2
		15194-1
		15194-2
		4914-1
		4914-2

Table 3: List of neuroblastoma tumor samples and tissue sections used in this study, two samples from each patient were provided

Brain tumor samples of 37 patients with different types and grades of brain tumor and 3 normal brain tissues were kindly provided by Assoc. Prof. Hasan Uğur Çağlar from School of Medicine, Department of Neurosurgery, Ankara University (Table 4)

Type and Grade	Brain Tumor	Type and Grade	Brain Tumor
Oligodendroglioma	O1	Glioblastoma	G2
Oligodendroglioma	O4	Glioblastoma	G4
Oligodendroglioma	O5	Glioblastoma	G6
Ependymoma	E9	Glioblastoma	G12
Pilocytic Astrocytoma	P12	Glioblastoma	G13
Astrocytoma3	A3/22	Glioblastoma	G14
Astrocytoma4	A4/41	Glioblastoma	G16
Astrocytoma4	A4/43	Glioblastoma	G17
Astrocytoma4	A4/49	Glioblastoma	G18
Astrocytoma4	A4/55	Glioblastoma	G19
Astrocytoma4	A4/56	Glioblastoma	G20
Astrocytoma4	A4/59	Glioblastoma	G21
Normal Brain	NR/61	Glioblastoma	G31
Normal Brain	NR/63	Glioblastoma	G32
Normal Brain	NG63	Glioblastoma	G33
Meningioma	M1	Glioblastoma	G34
Meningioma	M5	Glioblastoma	G36
Meningioma	M8	Glioblastoma	G37
Meningioma	M9	Glioblastoma	G40
Meningioma	M23	Glioblastoma	G42

Table 4: List of brain tumors of different types and grades used in this study

3.1.4 Cell Culture:

3.1.4.1 Cell Lines:

Two frozen neuroblastoma cell lines SK-NAS and CLB-MA1 (LT) were obtained from Dr. Valérie Combaret of Oncologie Génétique Centre Léon Bérard, France. Cells were tested and mycoplasma free. Cell lines in cryotubes were immediately stored in liquid nitrogen tanks after their arrival in dry ice.

3.1.4.2 Media and Solutions

Two cell lines SK-NAS and CLB-MA1 (LT) were cultured in 25 ml flasks (Greigner-Bio) as monolayer. Cell lines were grown in RPMI-1640 (Biological

Industries) supplied with 10% FBS (Sigma), 50mg/ml penicillin / streptomycin and non-essential amino acids (Biochrom AG). Cell lines were cultured at 37°C in an incubator with 5% CO2 (Heto-Holten, Surrey, UK). Cells were handled in sterile laminar hoods (Heto-Holten, Surrey, UK). Reagents were kept at 4°C except Trypsin – EDTA which was stored at -20 °C and pre-heated to 37°C before use.

3.1.4.3 Antibody

Rabbit polyclonal antibody to LSAMP (ab64427) was purchased from Abcam (Cambridge, USA).

3.2 Methods:

3.2.1 The cDNA Synthesis:

RevertAid^{TM} First Strand cDNA synthesis kit (#k1622) (Fermentas, USA) was used for all cDNA synthesis reactions. All reagents were provided by the kit. Total RNA samples isolated from brain tumor patients were reverse transcribed according to manufacturer's protocol. 2 µg of RNA template was mixed with 1 µl oligodT and DEPC treated water is added to 12 µl. Mixture was gently pipetted and incubated at 70°C for 5 minutes. After incubation 4 µl of 5X reaction buffer, 1 µl of Ribolock^{TM} Ribonuclease inhibitor and 2 µl of dNTP mixture were added in order. The mixture was gently pipetted and incubated at 37°C for 5 minutes. In next step, 1 µl of Revert Aid ^{TM} M-MuLV Reverse Transcriptase is added and incubated at 42°C for 1 hour proceeded by 10 minutes at 70°C. The products were stored at -20°C for further use.

3.2.2 Multiplex PCR Reaction:

Multiplex PCR (MP-PCR) was performed by using genomic primers for *LSAMP* and *GAPDH.* All PCR reactions were carried out by using Techne-512 PCR machine (Techne Inc). Optimal conditions were obtained by changing primer concentrations and temperature. Reaction conditions and final concentrations of reagents used in master mix for one tube (25 µl) are listed in Table 5 and Table 6, respectively. Templates containing 100 ng or 200 ng of DNA were used in reactions. All resulting PCR products were assessed by running in 2% agarose gels with EtBr and visualized under UV.

Reagent	**Stock Concentration**	**Final Concentration**	**Master Mix Setup**
Reaction Buffer	10 X	1 X	2.5 µl
LSAMPgnf54	10 pm	0.4 pm	1 µl
LSAMPex3R	10 pm	0.4 pm	1 µl
GAPDH_GENOMIC_20070206_F	10 pm	0.15 pm	0.375 µl
GAPDH_GENOMIC_20070206_R	10 pm	0.15 pm	0.375µl
MgCl2	50 mM	1.5 mM	0.75 µl
DyNAzyme™ II DNA Polymerase	2 U/µl	2 U	1 µl
ΔdH_2O	-	-	Add to 25 µl*

Table 5: MP-PCR master mix reagents and concentrations

Reaction Steps	Cycle Numbers
5 min. of initial denaturation at 95°C	1
30 sec. at 95°C	35
30 sec. at 60°C	
35 sec. at 72°C	
10 min. at 72°C	1

Table 6: MP-PCR Reaction conditions

3.2.3 Semi-Quantitative PCR Reaction:

Brain tumor cDNAs were tested for *LSAMP* expression level by semi Q-PCR method. *GAPDH* was selected as a housekeeping gene before starting to experiments and all samples were tested for *GAPDH* expression. All PCR reactions were done in Techne-512 PCR equipment (Techne Inc). Optimal conditions were obtained by changing primer concentrations and temperature. Reaction conditions and final concentrations of reagents used in master mix for one tube (20 µl) are listed in Table 7 and Table 8, respectively. All resulting PCR products were assessed by running in agarose gels with EtBr and visualized under UV.

Reagent	**Stock Concentration**	**Final Concentration**	**Master Mix Setup**
Reaction Buffer	5 X	1 X	4 µl
LSAMP Forward Primer#	10 pm	0.5 pm	1 µl
LSAMP Reverse Primer#	10 pm	0.5 pm	1 µl
Phire™ Hot Start DNA polymerase	-	-	0.4 µl
ΔdH_2O	-	-	Add to 20 µl*

Table 7: Semi-Q-PCR master mix reagents and concentrations

Reaction Steps	**Cycle Numbers**
30sec. initial denaturation at 98°C	1
5 sec. at 98°C	30
5 sec. at 63°C	
20 sec. at 72°C	
1 min. at 72°C	1

Table 8: Semi-Q-PCR Reaction conditions

3.2.4 Quantitative RT-PCR Reaction:

SYBR Green I method recommended by manufacturer was applied for analysis of *LSAMP* expression in brain tumors and normal brain tissue. *LSAMP* primer mix and Beta-actin gene (*ACTB)* primers were purchased from OriGene and used in all reactions. Beta-actin gene was used as internal control. 37 tumor and 3 normal brain samples were tested in duplicates for both *LSAMP* and beta-actin gene (*ACTB).* Reaction mixture setup for one tube and conditions are listed in Table 9, 10 and 11.

Corresponding readings of expression for both genes in tumors and normal samples were used in calculating expression differences (Table 12). The relative expression of *LSAMP* in tumors compared to normal brain tissues has been calculated by delta - delta CT method. The formula used in these calculations is as below:

- Relative Expression Ratio =

$$[E_{(LSAMP)}^{\Delta Ct_{LSAMP}(Ct\ normal\ -\ Ct\ tumor)} / E_{(ACTB)}^{\Delta Ct_{ACTB}(Ct\ normal\ -\ Ct\ tumor)}]$$

In this formula, $E_{(LSAMP)}$ and $E_{(ACTB)}$ represent the primer efficiencies of LSAMP and ACTB primers, respectively. Efficiency of the primers was approximated as 100% therefore a two fold increase at each cycle ($E_{(LSAMP)} = 2$, $E_{(ACTB)} = 2$). *ACTB* gene (B-actin) was used as reference for normalization. Ct normal values correspond to Ct values of normal brain samples, where Ct tumor belongs to Ct values of brain tumors. Results were log2 transformed and plotted (Fig.11). Statistical analysis of significance was performed by using one sample t-test at 95% confidence interval (H_0 = No difference exists in tumors compared to normal = 0).

Reaction Mixture	Volume
2X Master mix (Bio-Rad IQ)	12.5 µl
LSAMP primer mix	1 µl
Template cDNA	1 µl
dH2O	10.5 µl
Mineral oil	12.5 µl
Total	37.5 µl

Table 9: Q-RT-PCR reaction mixture for LSAMP gene

Reaction Mixture	Volume
2X Master mix (Bio-Rad IQ)	12.5 µl
Beta-actin forward primer (10pm)	1 µl
Beta-actin reverse primer (10pm)	1 µl
Template cDNA	1 µl
dH2O	9.5 µl
Mineral oil	12.5 µl
Total	37.5 µl

Table 10: Q-RT-PCR reaction mixture for beta-actin (ACTB) reference gene

Reaction Steps	Cycle Number
5 min. of initial denaturation at 95°C	1
15 sec. at 95°C	40
15 sec. at 62°C	
15 sec. at 72°C	
10 min. at 72°C	1
Melting Curve Step	
1 min. at 95 °C	1
30 sec. at 55 °C	
30 sec. at 95 °C	

Table 11: Reaction setup in quantitative RT-PCR experiments

3.2.5 Agarose Gel Electrophoresis

2% agarose gels with 30ng/µl EtBr were prepared to run PCR products. 2µl of 6X DNA loading dye was added to 10µl of each PCR product and gently pipetted. 1X TAE buffer was added into gel electrophoresis equipment and gel tray was placed properly. Gene Ruler DNA Ladder Mix of Fermentas (100-10,000bp) (Burlington, Canada) was used as size marker and loaded into the first well. Samples were loaded into separate wells which are horizontally aligned and run vertically under 100 V for approximately 30 minutes. Visualization and photography of DNAs were carried out by using Bio-Rad (California, USA) Transilluminator equipment under UV light of 340 nm wavelength.

3.2.6 Thawing and Culturing Cell Lines

Cryotubes were removed from liquid nitrogen tanks and thawed at 37°C. Lids of the tubes were carefully loosened and pressure inside the tubes was decreased. The cells were resuspended gently by using a pipette and 5ml of growth medium pre-heated to 37°C was added into each tube inside the sterile hood. The cells were centrifuged at

1500 rpm for 5 minutes. Supernatants were discarded and the pellets were resuspended in 5 ml growth medium to plate into 25 ml flasks. Flasks were stored in a humidified incubator at 37°C with 5% CO2. Culture mediums were refreshed in the next day.

3.2.7 Growth and Passaging of Cell Lines

Cells were examined under bright field microscope everyday for their confluency and culture mediums were refreshed. All culture mediums and enzymes were pre-heated to 37°C. Inside the sterile hood, the old mediums were vacuumed and the cells were washed twice with PBS. PBS was removed with vacuum and 0.5 ml of trypsin was added to each flask. Cells were incubated for 2-3 minutes at room temperature. Detachment of cells from the flask surface was confirmed via microscope and 5 ml of fresh RPMI 1640 (with supplements) medium was added to deactivate trypsin. Medium was pipetted gently to scatter cells from flask surface. Each cell line was divided into two by transferring 2.5 ml into new two 25 ml flask for each. 2.5 ml of medium was added into each bringing the total volumes to 5 ml. Flasks were kept in incubators at 37°C with 5% CO2.

3.2.8 Pelleting and Collecting Cells

The medium of flasks, where cells reached to optimal confluency, was aspirated and washed with PBS twice. 0.5 ml of trypsin was used for each flask to detach cells from the surface. Flasks were incubated at room temperature for 2-3 minutes. Fresh medium was supplied for each and gently pipetted. Suspensions were collected to 15 ml falcon tubes and centrifuged at 1400 rpm for 5 minutes. Supernatants were discarded and the pellets were washed with PBS twice. Tubes were centrifuged again at 1400 rpm for 5 minutes and resulting supernatants were aspirated via vacuum carefully. Pellets were immediately kept at -80°C.

3.2.9 Genomic DNA Isolation

Cell pellets were removed from -80°C refrigerator and thawed at room temperature. Qiagen DNeasy Tissue Kit (Venlo, The Netherlands) was used for isolating genomic DNA according to manufacturer's recommended protocol. Nanodrop Spectrophotometer (Nanodrop Technologies) was used for assessing the quality and amount of genomic DNA extracted. Samples were stored at -20°C.

3.2.10 Immunohistochemical Analysis of LSAMP Protein

Immunohistochemistry (IHC) was performed on sections of 6 neuroblastoma, 1 fetal kidney & suprarenal and 1 adult cerebellum tissues. Neuroblastoma tissues from patients were sectioned on poly-l lysine coated slides and were provided by Dr. Aylin Okçu Heper from School of Medicine, Department of Pathology, Ankara University. Dr. Emin Öztaş from Gülhane Military Medical Academy (GMMA) helped us with the IHC protocol. In brief, tissue sections were deparaffinized at 70°C and then in Xylene. After rehydration in graded alcohol series, glass slides were immerged in 10 mM citrate buffer, pH 6.0 and transferred into microwave for 20 minutes for antigen retrieval. Endogenous peroxidase was blocked by incubation of slides in 0.3 % H_2O_2 for 30 minutes. Phosphate buffered saline (PBS) was used in all washing steps. Tissue sections were incubated with LSAMP polyclonal antibody used at 1:500 dilutions in blocking solution, 50 µl was used for each slide. Slides were left overnight in humid chamber at 4 °C. Next day slides are washed with PBS and then universal staining kit (LabVision) was used according to manufacturer recommendations. Diaminobenzidine (DAB) was used as chromogen, and the slides were counterstained using Mayer's haematoxylin. Normal serum or phosphate buffered saline were used as negative controls, instead of the primary antibodies. Both positive and negative controls were processed in the same slides which have two distinct regions of tissue sections. Regions close to the slide ID was used as

positive where regions at the terminal ends were used as negative. Dark brown staining in sections was taken as positive reaction.

3.2.11 DNA extraction from Paraffin Embedded Tissues

5 µm sections of 12 paraffin embedded tissue samples collected from neuroblastoma patients were provided by Dr. Aylin Okçu Heper from Ankara University, Medical School, Department of Pathology. The same patients studied for LSAMP expression by using IHC were chosen. Boiling method protocol of Cao et al (2003) was used for DNA extraction from paraffin embedded tissues (Cao et al., 2003). Briefly, 1 ml of Xylene was added into the eppendorfs containing paraffin sections. Tubes were inverted several times and incubated a few minutes at room temperature. The samples were centrifuged for 5 min at 13,000 rpm to pellet the tissue. After obtaining pellets, the two steps, adding Xylene and centrifugation, were repeated. The pellets were washed by adding 100% ethanol, the tubes were inverted several times and centrifuged as 13,000 rpm for 5 min. The supernatant was removed and the step was repeated. 1 ml of 10 mM TE buffer was added into each tube and inverted several times before centrifuging at 13,000 rpm for 5 min. Supernatants were removed. 100 µl of 10 mM TE/1% Tween 20 containing 200 µg proteinase K was added into each tube and left to incubation at 55°C overnight. Next day, the tubes were heated at 97°C for 10 min to inactivate proteinase K and centrifuged at 13,000 rpm for 5 min. Supernatants were removed into new tubes and stored at - 20 °C.

4 RESULTS:

4.1 Genetic Analysis of LSAMP Gene Region in Tumors and Cell Lines:

Neuroblastoma genomic DNA was isolated from paraffin sections from 6 patients (2 sections for each) and 2 neuroblastoma cell lines; SK-NA-S and CLB-MA1, were analyzed for *LSAMP*. Samples 13598-1, 13598-2, 3755-1, 3755-2 and 3978-2 did not result in amplifications for both genes (data not shown). *GAPDH* was used as reference gene and *GAPDH* primers were used in combination with *LSAMP* primers in Multiplex-PCR reactions. 2 HCC (hepatocellular carcinoma) samples, SKHep-1 and Hep-3B, which have normal copy numbers of *LSAMP*, were used as positive controls. Products were loaded together with Gene Ruler DNA Ladder mix (100-10,000 bp) in 2% agarose gels under constant voltage of 100V about 30 min. The expected product sizes for amplified *LSAMP* and *GAPDH* genomic regions were 98bp and 143bp, respectively. Visualization with Bio-Rad Transilluminator, revealed the banding patterns corresponding to two genomic regions. Although, GAPDH was longer in base pairs, it was amplified in all samples, but LSAMP having smaller base pairs, which would be easier to amplify, was not detected to be fully amplified in a few samples. This suggested possible deletions in LSAMP genomic region in these samples. Thus, among 6 clinical patient samples, one possible homozygous deletion and one LOH in *LSAMP* region was identified.

4.1.1 Homozygous Deletion of LSAMP Gene:

Although amplification patterns of *GAPDH* region were similar for all patient samples, cell lines and controls, in patient #28231 any band corresponding to *LSAMP* gene could not detected which suggests a homozygous deletion of *LSAMP* genomic region (Fig.6).

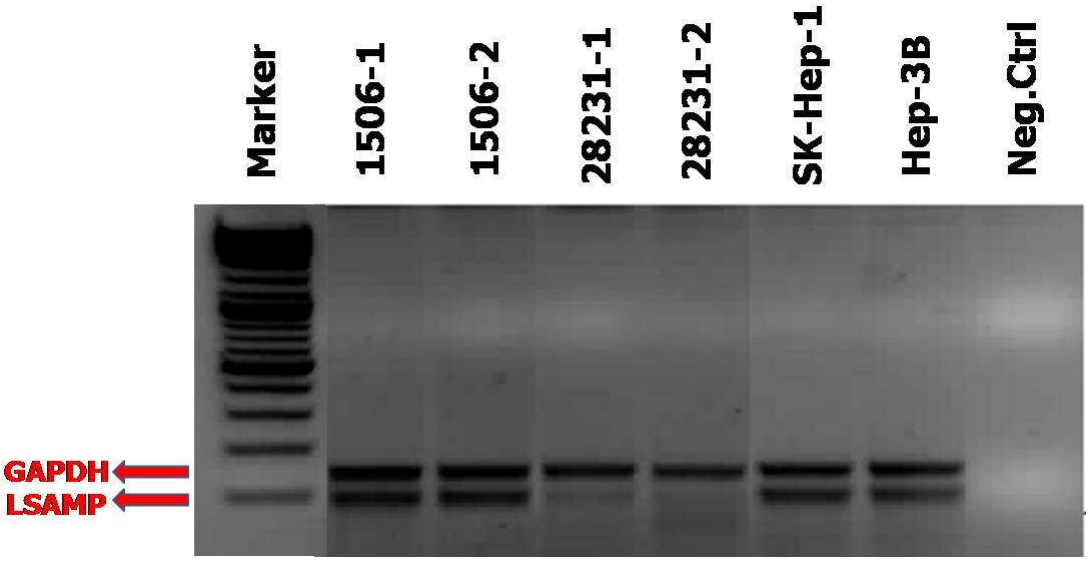


Figure 6: Homozygous deletion of LSAMP region in patient #28231 is shown. Multiplex-PCR result of LSAMP and GAPDH in neuroblastoma tumor and cell lines where HCC samples SK-Hep-1 and Hep-3B were used as positive control.

4.1.2 LOH at *LSAMP* Genomic Region:

Although, there was a band corresponding to *LSAMP* genomic region in tumor sample of patient #12116, the intensity of the band was weaker compared to *GAPDH* band. This result may suggest a possible LOH in correspondent *LSAMP* chromosomal region 3q13.31 (Fig.7).

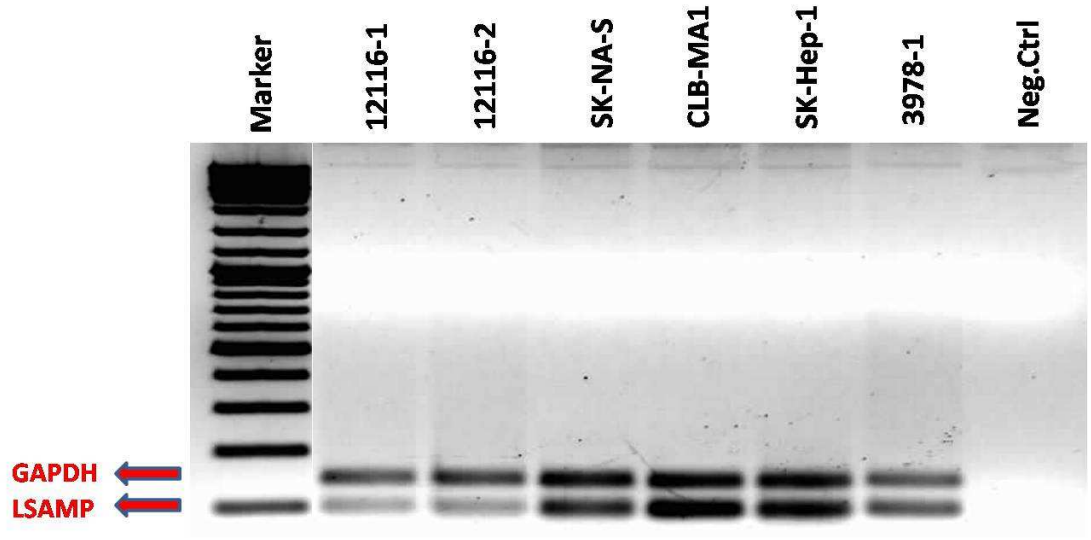
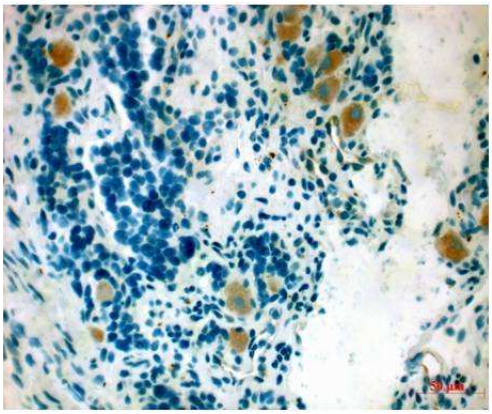


Figure 7: Possible LOH in LSAMP locus of patient sample #12116 is shown. Multiplex-PCR result of LSAMP and GAPDH in neuroblastoma tumor and cell lines where HCC sample SK-Hep-1 was used as positive control.

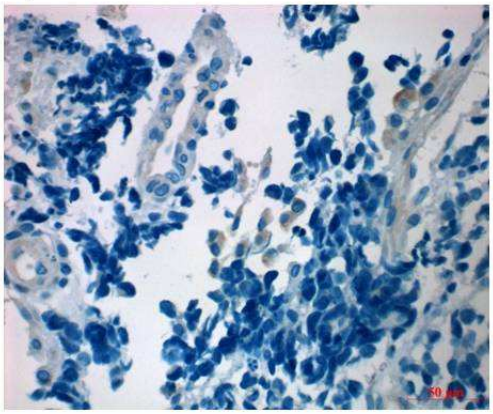
4.2 Assessment of LSAMP Protein in Neuroblastoma Tissues:

6 neuroblastoma tissues sectioned from the same patients analyzed for any genomic aberrations in *LSAMP* gene were further assessed at the protein level. IHC was used to detect LSAMP protein in neuroblastoma tissues. Additionally, healthy adult cerebellum and fetal kidney & suprarenal tissues were used as positive controls. Pictures of successful staining were shot with Zeiss Axiocam Imager A1 microscope (Gottingen, Germany). We observed a common weak staining pattern for all tumor samples compared to positive controls. Moreover, consistent to the result of genomic analysis, we did not see any protein in patient sample #28231 (Fig.8).

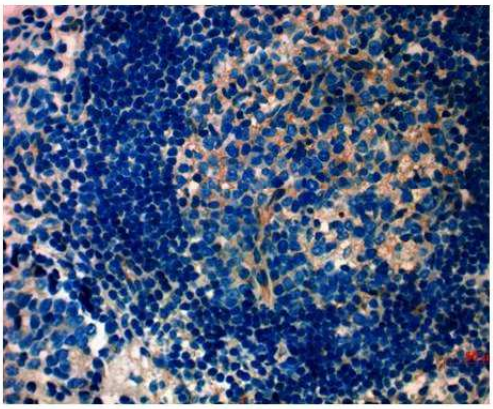
A) 1506



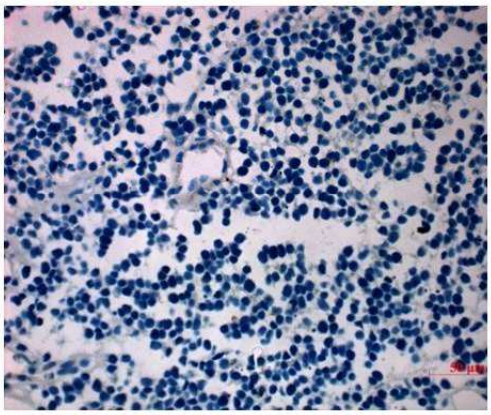
B) 3755



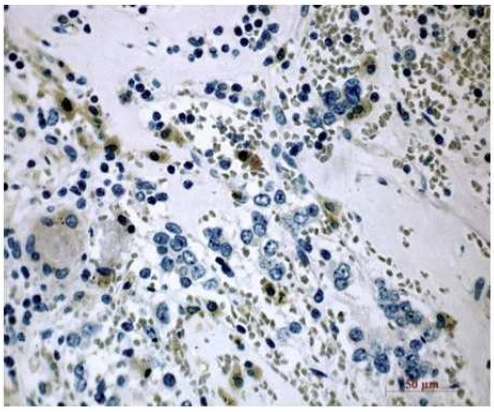
C) 3978



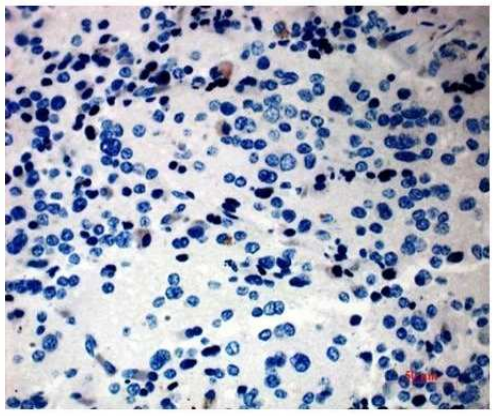
D) 28231



E) 12116



F) 13598



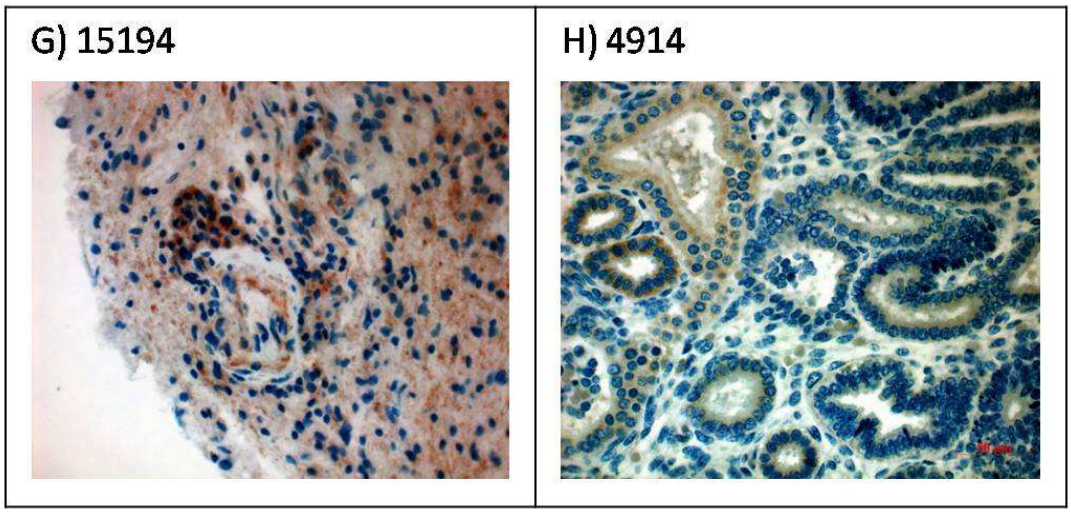


Figure 8: LSAMP protein levels in neuroblastoma tissues revealed by IHC (A, B, C, D, E, and F). Healthy adult cerebellum and fetal kidney &suprarenal tissue sections are used as controls (G & H).

4.3 Analysis of LSAMP Expression in Brain Tumors:

In addition to genomic and protein level analyses, we also assessed the expression of *LSAMP* in mRNA level in other tumors derived from neural tissues. A set of brain tumor patient RNAs were first reverse transcribed into cDNAs and tested for *GAPDH* gene levels which was used as reference gene in semi-quantitative PCR experiments (Fig.9 & Fig 10). All samples were used in analysis of *LSAMP* expression with semi quantitative PCR and Q-RT-PCR methods.

4.3.1 Semi Quantitative PCR Results:

All brain tumor samples were first tested by using primer pairs designed for amplifying a large region involving 6 exons in *LSAMP* gene. Glioblastoma samples were successfully amplified (Fig.10). Meningioma, oligodendroglioma, astrocytoma, ependymoma and pilocytic astrocytoma samples were further analyzed in nested PCR reactions by using primer pairs designed for amplifying a smaller region inside the first amplification (Fig .11).

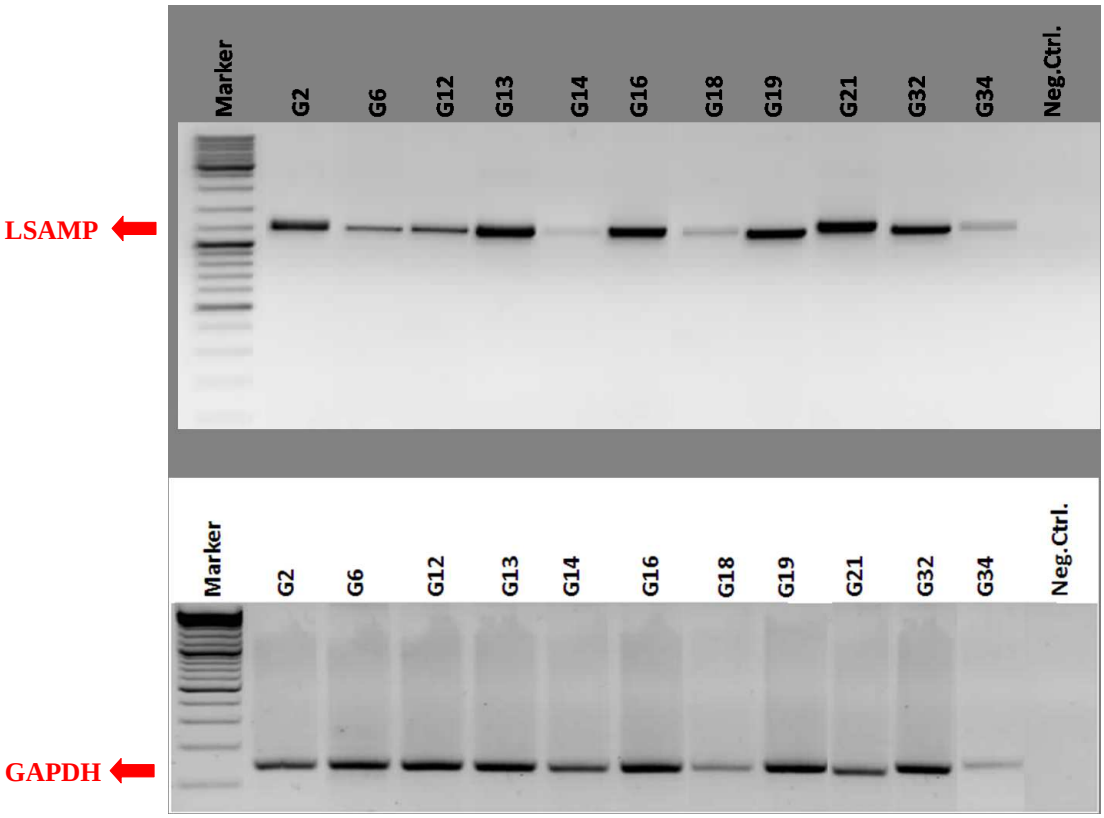


Figure 9: LSAMP and GAPDH expression levels of the same glioblastoma samples.

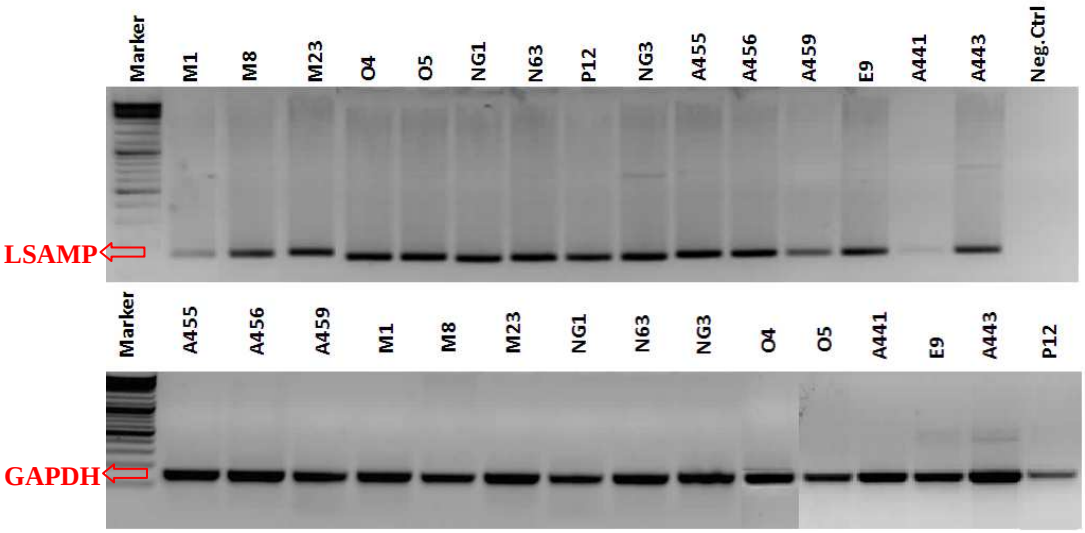


Figure 10: LSAMP expression in brain tumors; M series: Meningioma, O series: Oligodendroglioma, N series: Normal brain, P 12: Pilocytic Astrocytoma A series: Astrocytoma Grade 4, E9: Ependymoma.

Results showed us in some tumors although *GAPDH* is well expressed, *LSAMP* expression was lower compared to normal brain samples. A table summarizing these results showed us there is particularly a difference of expression in astrocytoma grade 4, meningioma and glioblastomas (Table 12). In this table, weak intensity bands are represented by "+", medium intensity by "++" and high intensity by "+++" signs. Although, semi quantitatively we observe the decrease in *LSAMP* expression, a more precise method Q-RT-PCR was performed in next step for detecting actual expression levels.

4.3.2 Q-RT-PCR Detection of *LSAMP* Expression in Brain Tumors:

We performed Q-RT-PCR experiments by using commercially obtained *LSAMP* primers amplifying a small region between 100-140bp and Beta-actin primers to use Beta-actin expression as control. BioRad SyBr Green Kit was used to prepare master

mix. The results were analyzed by using delta - delta Ct method and indicate a clear decrease in *LSAMP* expression in majority of meningiomas, astrocytoma grade 4 and glioblastoma tumors compared to normal brain tissue (Table. 13, Fig.11).

The calculated fold changes suggesting *LSAMP* has generally lower values for tumors compared to normal seem statistically significant at 95% confidence interval (p-value=0.002).

Type and Grade	Brain Tumor	LSAMP	GAPDH	Type and Grade	Brain Tumor	LSAMP	GAPDH
Oligodendroglioma	O1	No	+‡	Glioblastoma	G4	No	+
Oligodendroglioma	O4	+++	+++	Glioblastoma	G6	+	+++
Oligodendroglioma	O5	+++	++	Glioblastoma	G12	+‡	+++
Ependymoma	E9	++	++‡	Glioblastoma	G13	+++	+++
Pilocytic Astrocytoma	P12	No	+‡	Glioblastoma	G14	+	++
Astrocytoma3	A3/22	No	+	Glioblastoma	G16	+++	+++
Astrocytoma4	A4/41	++	+++	Glioblastoma	G17	No	+
Astrocytoma4	A4/43	++	+++	Glioblastoma	G18	+	+
Astrocytoma4	A4/49	+	+++	Glioblastoma	G19	+++	+++
Astrocytoma4	A4/55	+++	+++	Glioblastoma	G20	No	‡
Astrocytoma4	A4/56	+++	+++	Glioblastoma	G21	+++	+++
Astrocytoma4	A4/59	+	+++	Glioblastoma	G31	No	+++
Normal Brain	NR/61	++	+++	Glioblastoma	G32	+++	+++
Normal Brain	NR/63	+	+++	Glioblastoma	G33	+++	+++
Normal Brain	NG3	+++	+++	Glioblastoma	G34	+	+++
Meningioma	M1	No	+++	Glioblastoma	G36	+++	+++
Meningioma	M5	No	++‡	Glioblastoma	G37	+++	+++
Meningioma	M8	++	+++	Glioblastoma	G40	No	+
Meningioma	M9	No	++‡	Glioblastoma	G42	No	++
Meningioma	M23	No	++	Glioblastoma	G42	No	++
Glioblastoma	G2	++	++	Glioblastoma	G42	No	++

Table 12: The relative expression levels of LSAMP and GAPDH in different types and grades of brain tumors revealed by semi quantitative PCR

TumorSamples	Ct $_{LSAMP}$ *	Ct$_{ACTB}$ **	Fold Change	LOG2
A322	30.62	30.2	0.737774	-0.43875
A441	23.735	16.9925	0.009219	-6.76125
A443	22.395	14.9	0.005472	-7.51375
A449	24.555	19.935	0.040142	-4.63875
A455	20.39	14.905	0.02204	-5.50375
A456	19.025	15.19	0.069168	-3.85375
A459	23.125	24.74	3.023564	1.59625
E9	22.515	24.085	2.93071	1.55125
G12	27.13	17.83	0.001566	-9.31875
G13	23.365	17.035	0.01227	-6.34875
G14	26.23	22.625	0.081123	-3.62375
G16	24.16	18.54	0.020071	-5.63875
G17	27.075	29.395	4.928846	2.30125
G18	27.165	27.25	1.046992	0.06625
G19	23.595	18.335	0.02576	-5.27875
G2	25.01	19.275	0.018533	-5.75375
G20	30.025	31.59	2.92057	1.54625
G21	22.48	17.34	0.027994	-5.15875
G31	24.695	24.725	1.007828	0.01125
G32	24.53	18.57	0.015857	-5.97875
G33	23.405	17.47	0.016134	-5.95375
G34	25.345	20.22	0.028286	-5.14375
G36	22.94	17.24	0.018988	-5.71875
G37	24.525	17.58	0.008011	-6.96375
G4	29.09	30.38	2.413706	1.27125
G40	27.83	24.09	0.073876	-3.75875
G42	26.265	23.435	0.138816	-2.84875
G6	28.36	20.04	0.003089	-8.33875
M1	26.77	24.955	0.280534	-1.83375
M23	30.73	21.495	0.001638	-9.25375
M5	23.71	19.595	0.056966	-4.13375
M8	26.485	17.455	0.001888	-9.04875
M9	27.205	21.38	0.017412	-5.84375
O1	28.415	25.325	0.115924	-3.10875
O4	21.735	19.965	0.289423	-1.78875
O5	23.695	24.0875	1.295716	0.37375
P12	26.895	28.735	3.533873	1.82125

Table 13: Quantitative RT-PCR results of LSAMP in brain tumors (*Ct normal brain $_{LSAMP}$: 23.0363, ** Ct normal brain $_{ACTB}$: 23.055)

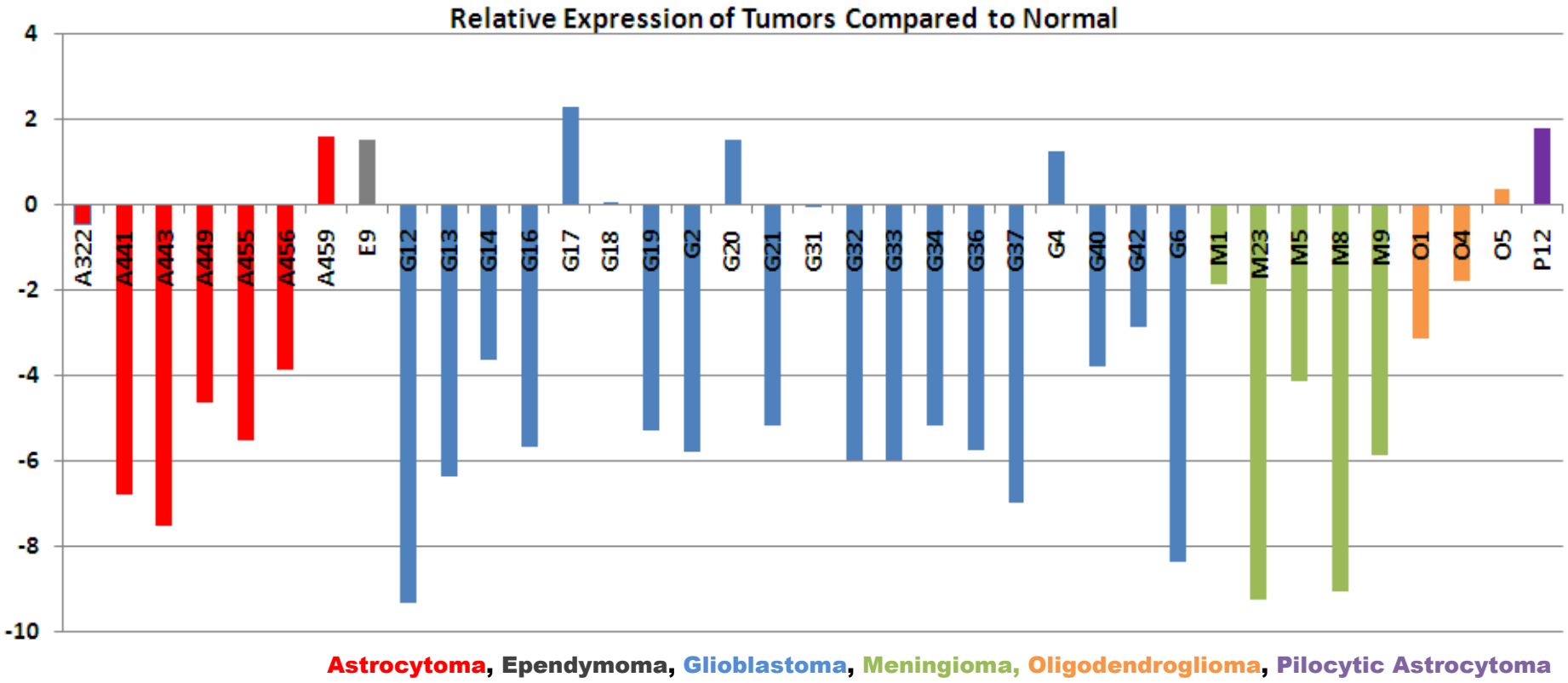


Figure 11: Expression differences in brain tumors relative to normal brain tissue.

5 DISCUSSION:

Risk stratification is a crucial step in prognosis of tumors and in the case of neuroblastoma, the most common extracranial solid tumor in children, it is far more important because of the heterogeneous nature of the tumor . Clinical studies showed that the good prognosis is highly correlated with increased survival in neuroblastoma patients. Therapeutic applications are needed to be performed in accordance with the stage of neuroblastoma for successful results. The unexplained behavior of the disease in different sub groups may result in ineffective therapies. Clinical observations alone are not successful to determine risk groups for many cases and the importance of genetic research in neuroblastoma emerged in recent years. Researchers have been focused on elucidating the molecular biology of neuroblastoma development to find potential tumor markers for improved prognosis and to answer the heterogeneous behavior of the disease. Analysis of patients revealed some genetic abnormalities that are seen frequently in most cases like *MYCN* amplification, 1p deletion and 17 q gain. Furthermore, translocation and methylation events of important regulator genes are observed for a number of cases (Maris and Matthay, 1999). However, the exact mechanism of tumor development is still unanswered and a model of neuroblastoma tumorigenesis via three different pathways has been hypothesized (Fig.3).

As a typical event observed in carcinogenesis, inactivation of a tumor suppressor gene in a neuroblast cell has been proposed for the initial step of the neuroblastoma development. Thus, identification of tumor suppressor genes has a great potential for revealing the mechanism of tumor development and further investigation of new therapeutic approaches. Compared to the current therapies involving surgery and radiation, specific gene targeted approaches would be more efficient and less destructive. Several ways exist which result in chromosomal aberrations that deactivate tumor suppressors such as deletions, mutations and epigenetic

modifications. Moreover, certain types of aberrations in chromosomal regions may be used as a characteristic marker of the tumors which results in better prognosis.

In this study, *LSAMP* was assessed as a potential tumor suppressor gene for neuroblastoma by using semi-quantitative PCR and IHC methods. PCR method was chosen for quick and accurate evaluation of *LSAMP* gene dosage in neuroblastoma tissue samples and cell lines where IHC was preferred for protein analyses in tissue sections. Among analyzed 12 samples, a total of 4 samples were identified as having *LSAMP* homozygous and heterozygous deletions compared to *GAPDH* internal controls and positive controls of SKHep-1 and Hep-3B (Table 14). However, the heterozygous banding pattern of the two samples might be due to the amplification of surrounding normal tissue genomic DNA.

Sample ID	**Loss**	**Sample ID**	**Loss**
12116-1	LOH	1506 -1	None
12116-2	LOH	1506 -2	None
28231-1	Homo. Del.	3755-1	None
28231-2	Homo. Del.	3755-2	None
3978-1	None	SK-NA-S	None
3978-2	None	CLB-MA1	None
13598-B3 -1	None	SK-Hep-1	None
13598-B3 -2	None	Hep-3B	None

Table 14: Multiplex-PCR analysis of LSAMP in neuroblastoma tissues showing possible deletions

IHC was performed by using rabbit polyclonal LSAMP antibody on neuroblastoma tissue sections obtained from same patients whose DNA was used for PCR experiments. IHC experiments showed us localized weakly stained areas for LSAMP protein in all of the samples. Two samples were used as positive controls where *LSAMP* is known to be well expressed. Samples 15194 (adult cerebellum) and 4914 (fetal kidney & suprarenal) revealed broader and stronger staining patterns compared to the tumor samples. Among tumor samples, 1506 and 3978 showed a stronger positivity compared to others (Fig.8).

Both genomic analysis and protein analysis suggests that there may be an association with *LSAMP* and neuroblastoma. Furthermore, the two results can be compared and a correlation may be established between patients that have genomic deletions of *LSAMP* also have lower protein expressions compared to patients with no loss.

An interesting result is that although LSAMP is known as a membrane protein, the staining pattern of the samples suggests a possible role of LSAMP also in cytoplasm. This observation may be correlated to the mechanism of IgLONs forming heterodimers with each other, a structure called “Diglon” (Reed et al., 2004). LSAMP might be dimerizing with OPCML, NEGR1 or NTM in cytoplasm and then functionally inserted into the plasma membrane. Also the existence of LSAMP isoforms with different functions yet unknown might be the result of staining spread through the cells. The formation of Diglons may suggest checking for other IgLON members in neuroblastoma since deletion of LSAMP may also affect the function of other IgLONs and consequently the signaling pathways they are responsible for. The positive correlation between IgLON family members was previously described for epithelial ovarian carcinoma where *LSAMP*, *OPCML* and *NEGR1* were decreased in expression coordinately. Moreover, *LSAMP* has been also declared for a strong predictor of poor outcome in epithelial ovarian cancers (Ntougkos et al., 2005).

Originating from the sympathetic nervous system, tumors may develop in any site in the lineage like adrenal medulla which is the most common region. *LSAMP*, as the name indicates, is tightly associated with limbic system and sympathetic system. It is highly expressed along the nervous system and plays an important role during neural development. Tumor suppressor genes are generally classified into two groups as some tumor suppressors are specific to certain tissues while some of them act as a tumor suppressor in multiple types of cancer arising from the same lineage. Thus, possible tumor suppressor function of *LSAMP* may also exist in brain tumors as well as neuroblastoma. We performed semi-quantitative PCR and Q-RT-PCR analyses for *LSAMP* in a set of brain tumors with different types and grades. In a majority of the samples tested, downregulation of *LSAMP* compared to normal brain was observed. Semi-quantitative and quantitative results were also found to be generally in accordance with each other. In a previous study Reed et.al (2007) also identified *OPCML* downregulation in brain tumors (Reed et al., 2007). Taken together, these two correlated results suggested *LSAMP* to be an important regulator in neural system tumors and a possible tumor suppressor for neural tumors (Fig.9, 10, 11 and Table 12).

OPCML were shown to be inactivated via LOH and CpG island methylation in epithelial ovarian carcinoma (Sellar et al., 2003). Inducing expression of OPCML in cell lines resulted in decreased proliferation and enhanced aggregation, which imply for a potential tumor suppressor role.

LSAMP was also shown to be deleted and epigenetically inactivated in CCRCC (Chen et al., 2003). Furthermore, inducing expression of fluorescently tagged *LSAMP* in cell lines suppressed cell proliferation and resulted in growth inhibition. Similarly, two recent studies declared *LSAMP* as a tumor suppressor for osteosarcomas revealed by SNP and CGH arrays (Kresse et al., 2009; Yen et al., 2009b) . In both cases, frequent homozygous and heterozygous deletions in chromosome region 3q13.31 were recorded and the low expression of LSAMP was

shown to be correlated with poor outcome (Kresse et al., 2009; Yen et al., 2009b). In the outcome data of Kresse et al., a positive association of *LSAMP* and *OPCML* was Also shown. In 7 of 9 tumor samples and 5 of 10 cell lines inspected, both chromosomal regions 3q13.31 and 11q25 of *OPCML* were deleted (Kresse et al., 2009).

In consequence, despite the high expression of not only *LSAMP* but also IgLON family members in neural tissues, their role in neural system tumors have not been researched in detail. The accumulating data associating them to different cancer types via LOHs and epigenetic modifications support the model of tumor suppressor inactivation in carcinogenesis. There is a high possibility that these genes are also inactivated in neuroblastoma as suggested by this study for *LSAMP* and their potential dimerization with each other. We observed heterozygous and homozygous deletions were observed in 2 of 6 clinical cases analyzed and in addition decreased or lost protein expressions compared to controls in all 6 samples were detected. Moreover, our data from genomic and protein analyses were found to be consistent with each other. *LSAMP* was also found to be downregulated in tumors derived from neural linage. All of these results suggest *LSAMP* may be a tumor suppressor in neural tumors and loss of *LSAMP* may be used as a marker. In this manner promising results of this study provides a preliminary step for further inspection of *LSAMP* and other IgLONs in neuroblastoma and other neural system tumors.

6 Future Perspectives:

Although, a potential tumor suppressor role of *LSAMP* was observed in this study and suggested by data in literature, it would be wise to conduct further experiments for confirmation of the results. As a first step to confirm deletions, using advanced techniques like PCR amplification of polymorphic markers following capillary electrophoresis and CGH arrays can produce more detailed analysis revealing any genomic variations in the samples by comparing to normal tissues.

As a common event observed in tumor suppressor inactivation during tumorigenesis, epigenetic modifications in the *LSAMP* promoter would be responsible for its decreased protein levels in samples we tested. Methylation status can be checked by performing methylation specific PCR reactions, easily.

A further step to evaluate *LSAMP* as a tumor suppressor would be functional analysis in cell lines. The affect of *LSAMP* expression in cell lines would provide us valuable and strong data about its function in tumorigenesis. *LSAMP* expression can be induced by using expression vectors for cell lines and any inhibitory effect can be recorded. Moreover, at the protein level cellular expression and localization of LSAMP can be tracked via the use of fluorescent tags like GFP.

The sample size of the experiments may be increased for increasing the statistical significance of the data. We could not study expression of *LSAMP* in neuroblastoma because of the lack in patient samples. Obtaining these cDNAs from neuroblastoma patients and Q-RT-PCR analysis of expression in different grades of tumor would be also valuable. In order to find a correlation of *LSAMP* and different neuroblastoma stages, the samples from different stages should be used to detect its association to good or poor outcome .Other genomic aberrations may also have a negative or

positive correlation to *LSAMP* deletions. The genomic status of different factors like MYCN amplification status should be checked together for better assessment of *LSAMP* gene in neuroblastoma.

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